Threonine in the selectivity filter of the acetylcholine receptor channel

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ABSTRACT The acetylcholine receptor (AChR) is a cation selective channel whose biophysical properties as well as its molecular composition are fairly well characterized. Previous studies on the rat muscle α-subunit indicate that a threonine residue located near the cytoplasmic side of the M2 segment is a determinant of ion flow. We have studied the role of this threonine in ionic selectivity by measuring conductance sequences for monovalent alkali cations and bionic reversal potentials of the wild type ($\alpha\beta\gamma\delta$ channel) and two mutant channels in which this threonine was replaced by either valine (αT264V) or glycine (αT264G). For the wild type channel we found the selectivity sequence Rb > Cs > K > Na. The αT264V mutant channel had the sequence Rb > K > Cs > Na. The αT264G mutant channel on the other hand had the same selectivity sequence as the wild type, but larger permeability ratios P_x/P_{Na} for the larger cations. Conductance concentration curves indicate that the effect of both mutations is to change both the maximum conductance as well as the apparent binding constant of the ions to the channel. A difference in Mg^{2+} sensitivity between wild-type and mutant channels, which is a consequence of the differences in ion binding, was also found. The present results suggest that αT264 form part of the selectivity filter of the AChR channel were large ions are selected according to their dehydrated size.

INTRODUCTION

In spite of the progress provided by the application of recombinant DNA techniques, which allow one to modify the structure of proteins, their use to study ions channels has been restricted almost exclusively to characterize amino acid residues involved in a particular function. A relationship between such a function and the nature of the amino acids involved has seldom been studied and only in few cases a mechanistic interpretation of the mutated channel properties has been attempted (Konno et al., 1991; Yool and Schwarz, 1991).

The acetylcholine receptor channel (AChR) is a protein in which amino acid residues involved in either ion conduction (Mishina et al., 1985; Imoto et al., 1986, 1988) or gating properties (Mishina et al., 1985) have been characterized in great detail making this channel a convenient molecule to study structure function relationships. Conduction properties of the native AChR have been characterized in several preparations. The receptor channel is permeable to monovalent as well as divalent cations (Adams et al., 1980). In most preparations, permeability for monovalent cations follows the water mobility (Hille, 1984; Quartararo et al., 1987; Konno et al., 1991) as for the divalent cations the opposite has been found. The conductance sequence measured by single-channel recording is different from the permeability sequence (Quartararo et al., 1987, Konno et al., 1991). Small organic cations are able to pass through the channel. (Dwyer et al., 1980). The factor which determines permeability was found to be the size of the organic ion and not its particular chemical nature. From this measurements the narrowest section of the pore was postulated to have an area of 6.5 × 6.5 Å (Dwyer et al., 1980). As for its length, streaming potential measurements suggest that it is as short as 3-6 Å (Dani, 1989). The molecular nature of this narrow regions as well as the mechanism of ion selection is still unknown.

Labeling studies with noncompetitive inhibitors (NCI) which block ion flux primarily by promoting desensitization have yielded inconsistent results with respect to the involvement of different domains in forming the channel walls (see Karlin, 1991). This is most likely due to the fact that NCI's are a heterogeneous class of compounds and that the mode of action of NCI's used for labeling is rather unclear. The presumed "open channel block" of the various NCI's used for labeling studies has not been demonstrated yet unequivocally.

From the study of mutant channels, a more consistent picture has been found suggesting that a domain, present in all four subunits of the AChR channel, designated the M2 segment (Noda et al., 1983) and the adjacent "bends," are involved in ion conduction and perhaps in the formation of the channel pore (Imoto et al., 1986, 1988; Leonard et al., 1988). There are two motifs which have been shown to be critical for ion conduction and

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open-channel block (see Dani, 1990, for review). These are negatively charged amino acids, which may form a ringlike structure at the channel's entries (Imoto et al., 1988), and amino acid residues that contain hydroxyl groups in their side chain, thought to be lying in the wall of the channel pore, (Leonard et al., 1988; Charnet et al., 1990; Villarroel et al., 1991). Some of these domains should contribute, at least partly, to the selectivity filter of the channel.

Studies on the fetal form of the AChR channel ($\alpha\beta\gamma\delta$) from rat skeletal muscle suggest that a threonine residue located near the cytoplasmic end of the transmembrane segment M2 (αT264) is involved in controlling the ion flow through the pore (Villarroel et al., 1991). Here we report the biophysical properties of two mutant channels (αT264V and αT264G) constructed to elucidate the role of αT264 in determining conductance and selectivity of the rat muscle AChR channel.

MATERIALS AND METHODS

Mutant channels

The wild type of the fetal form of rat AChR $\alpha\beta\gamma\delta$ (Witzemann et al., 1990) and two mutant channels in the α-subunit were studied. The mutant channel in which the threonine at position 264 of the α-subunit has been substituted by valine is referred as αT264V. The one that carries a glycine at that position is referred as αT264G. The construction of αT264V and αT264G mutant channel has been described in Herlitze and Koenen (1990), and in Villarroel et al. (1991).

Oocyte injection

Xenopus oocytes were injected with RNA encoding for the four subunits ($\alpha\beta\gamma\delta$) of the fetal form of the AChR channel (Witzemann et al., 1991) with the wild type or mutated α-subunit. Oocytes were incubated in OR-2 medium (82.5 mM NaCl, 2.0 mM KCl, 1.0 mM $MgCl_2$, and 5 mM Hepes, pH 7.5) with Penicillin, Streptomycin and Cefuroxim (Hoechst AG, Frankfurt) added at 100 units/ml at 19°C. One day after injection the oocytes were incubated in 1 mg/ml collagenase (type I; Sigma Chemical Co., St. Louis, MO) for 30 min at room temperature. After collagenase treatment the follicle cell layer was removed mechanically with forceps (Methfessel et al., 1986). Three days after injection oocytes were tested for the presence of ACh receptors.

The responses to 1 μM acetylcholine were tested using conventional two electrode voltage clamp (Methfessel et al., 1986) in normal frog Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM Hepes, pH 7.2, 240 mOsm). Electrodes filled with 3 M KCl and a resistance of 1 MΩ and 0.1–0.2 MΩ were used to set the voltage and measure current, respectively. Most of the oocytes responded to acetylcholine applications with a current of 8–10 μA at a holding potential of −70 mV.

Patch clamp

Inside-out patches isolated from the oocyte membrane were obtained using standard patch-clamp methods (Hamill et al., 1981; Methfessel et al., 1986). Borosilicate glass pipettes (Hilgenberg, Malsfeld, inner diameter 2 mm, outer diameter 5 mm) were pulled in two stages using a L/M-3P-A puller (List Electronics, Darmstadt), covered with Sylgard, and fire polished immediately before use. Pipettes had a tip resistance of 5 MΩ when filled with 100 mM KCl solution. The solution used was made of the permeant cation (NaCl, KCl, RbCl, CsCl or NH_4Cl) in different concentrations, 10 mM Hepes, and 10 mM EGTA adjusted to pH 7.2 with the corresponding hydroxide form of the permeant cation. In solutions of low Cs^+ concentrations both Hepes and EGTA was lowered to 2 mM. The activity of K^+ containing solution was measured with a K^+ electrode (Radiometer, Copenhagen). Reversal potentials were measured using Na^+ as a reference cation in the bath and the test cation in the pipette. After an inside-out patch was formed, single-channel currents were measured at different potentials around the reversal potential. Immediately after that, the patch was broken by applying positive pressure and the potential of the pipette, nominally zero, was measured. Because the application of negative pressure during seal formation may contaminate the pipette tip solution, only measurements on patches isolated without negative pressure were accepted. Current amplitudes were pooled in 2 mV bins and a current voltage I–V curve was constructed. Then, a 5th order polynomial was fitted to the experimental points and used to interpolate the zero current potential. The measured pipette potential was substracted from this potential. The zero current potential was corrected by the liquid junction potential between the two solutions, calculated using the ion mobilities (Hille, 1984; Quartararo et al., 1987), to obtain the reversal potential. The temperature was 18°C.

Fit procedures

To fit the data to the model the nonlinear minimization algorithm AJUSTE was used (Alvarez et al., 1991). For a given model the program finds the parameters that minimizes the sum of the square of the difference between experimental and model values. The output of the program is the set of parameter that produces the best fit together with the standard deviation of the measurement.

THEORY

Saturation and surface charge

In a simple one-site one-ion channel the conductance follows a Michaelis-Menten relationship with apparent dissociation constant K_s and maximum conductance G_s (Hille, 1984). If the ion concentration in the neighborhood of the channel mouth is (Cs) then the conductance is given by:

$$G[(Cs)] = G_s(Cs)/[K_s + (Cs)]. \quad (1)$$

The presence of fixed charges in the vestibules of the channel perturbs the local ion concentration. There are several ways to account for this. A simple one is the Gouy-Chapman theory (McLaughlin et al., 1971) in which a surface charge of density σ produces a surface potential ϕ_s related to the ion species j of valence z_j at concentration $(C)_j$ as:

$$\sigma^2 = 2\epsilon\epsilon_0 kT\Sigma(C)_j[\exp(-z_j\phi_s) - 1], \quad (2)$$

where ϵ, ϵ_o, k and T are the permittivity of the free space, the dielectric constant of the solution, the Boltzmann

constant and the absolute temperature. The local ion concentration is given by $(Cs)_L = (Cs) \exp(-z_j \phi_s)$. This expression was inserted in Eq. 1 and was used to fit the conductance concentration curves. The surface charge density was expressed as one elementary charge e distributed in a circular area of radius R ($\sigma = e/\pi R^2$). For conductance measured at negative potentials this corresponds to surface charge of the external vestibule. This model is the simplest one that contains the two features, saturation of current at high concentration and the effect of charged vestibules. Though more complex models are available (Dani, 1989; Konno et al., 1991), we chose the simplest one because of the smaller number of free parameters.

Permeability

Permeability is defined as the ratio between ion flux and concentration. If the ion flux is expressed as conductance at a given voltage, at low concentration where conductance is proportional to concentration (Eq. 1) the permeability is given by:

$$P = G_s/K_s. \tag{3}$$

This relation can also be obtained from first principles, or from diffusion theories (see Hille, 1984). In that case permeability is also the product of binding and mobility of the ion (Eisenman and Horn, 1983).

Permeability ratios

In a bionic situation where Na^+ is present in the bath and a monovalent ion X is present in the pipette, both at the same concentration, a zero current potential V_{rev} is measured. This potential defines the permeability ratio P_x/P_y (Hille, 1984) by:

$$V_{rev} = (RT/F)\mathrm{Ln}(P_X/P_{Na}), \tag{4}$$

where R, T, and F are the gas constant, Faraday's constant and absolute temperature, respectively.

RESULTS

The AChR α-subunit has been postulated to have four transmembrane segments (Fig. 1 *A*) (Noda et al., 1983). From these the M2 segment is believed to be important for ion conduction. The M2 segment of α-subunit in the rat fetal AChR receptor has a high degree of homology with the M2 segment of other subunits (Witzemann et al., 1991), as indicated by boxes in Fig. 1 *B*. Mutational analysis in *Torpedo* AChR identified clusters of charged

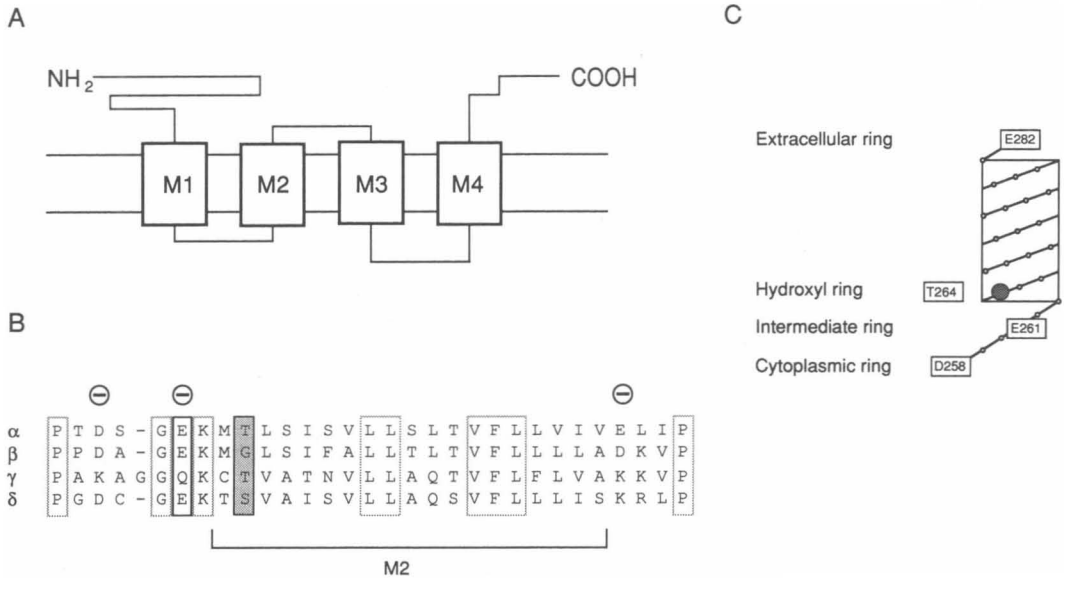

FIGURE 1 The M2 segment of the α-subunit ACh receptor channel. (*A*) shows the transmembrane folding of the α-subunit protein. The putative transmembrane segments are indicated as M1–M4. (*B*) shows the amino acid sequence of the M2 segment of four subunits in single-letter code. Notice the high degree of homology indicated by boxed amino acids. The shaded amino acids indicate the position of αT264, and the homologous amino acids in the other subunits. The minus sign indicates the position of the amino acids contributing to the anionic rings. (*C*) shows an α-helix representation of the M2 segment α-subunit showing the location of the αT264 residue, near the cytoplasmic end of the M2 segment close to the intermediate ring of negative charges. Numbers refer to those given in Witzemann et al. (1990).

amino acids which may form a structure of anionic rings on both sides of the membrane as indicated in Fig. 1 *B*. In an α-helix representation of the putative transmembrane segment M2 of the α-subunit (Fig. 1 *C*), the threonine 264 is located next to the intermediate anionic ring (Fig. 1, *B* and *C*). In the γ- and δ-subunits also polar amino acids are found at homologous positions (*shaded*). The effect on ion conductance and permeability of two point mutations at this position will be described. These are a substitution of this threonine residue by either valine (αT264V) or glycine (αT264G). Valine has similar size as threonine (Richards, 1974) but it lacks the hydroxyl group, whereas glycine is much smaller in size.

Mutation of αT264 increases or decreases channel conductance depending on the volume of the side chain

As shown previously, the channels harboring the αT264V or αT264G mutations are functional (Villarroel et al., 1991). Fig. 2 *A* shows single-channel currents of wild type (WT) and both αT264V and αT264G mutant channels measured in symmetrical Cs^+ solution in absence of divalent cations. The single-channel current of the αT264V mutant channel has about a half of the amplitude of the wild-type channel (Fig. 2 *B*), whereas the αT264G mutant channel has a somewhat larger single-channel current amplitude. None of the mutations produced any obvious change in the average duration of single channel currents.

Conductance changes for αT264 mutations are larger for larger ions

Current carried by a large ion like Rb^+ is more affected by the αT264V mutation than the current carried by Na^+. Fig. 3 *A* shows a comparison between I–V curves of wild type and the αT264V mutant channel when Rb^+ is the permeant ion. A decrease in conductance of ~ 35% can be seen at all voltages, being more pronounced at negative voltages. On the other hand, when current is carried by Na^+ there is only a small decrease in conductance of < 20% (Fig. 3 *B*). The replacement of αT264 by valine decreases channel conductance for both inward and outward currents. No particular change in the shape of the current voltage curve is found for Na^+ whereas for Rb^+, the mutation transform the almost linear current voltage curve of the wild type channel into an hyperbolic curve. Such curves are found when the rate limiting step of ion permeation is strongly voltage dependent.

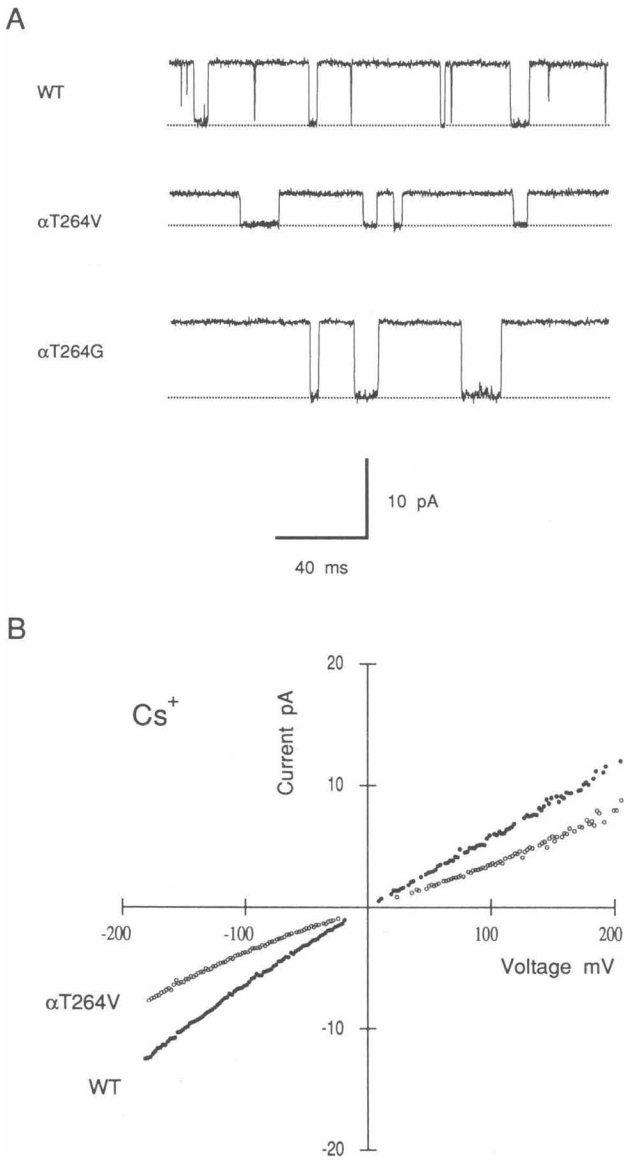

FIGURE 2 Differences in single-channel conductance between wild-type and mutant channels. (*A*) Single-channel currents of the wild type, αT264V and αT264G mutant channels. The αT264V mutant channel has a conductance of about half the value for the wild type channel. For the αT264G mutant channel a conductance 15% larger than the wild type is seen. The applied voltage was −100 mV. (*B*) Current-voltage curves of wild type and αT264V mutant channel. Notice the reduction of conductance in the whole range of voltages. Currents measured in 100 mM Cs^+, 10 mM Hepes and 10 mM EGTA, pH 7.2.

Conductance ratios and reversal potentials

Both αT264V and αT264G mutations modify the channel conductance of the AChR in larger proportions for large ions (Fig. 4 *A*, Table 1). Because different measure-

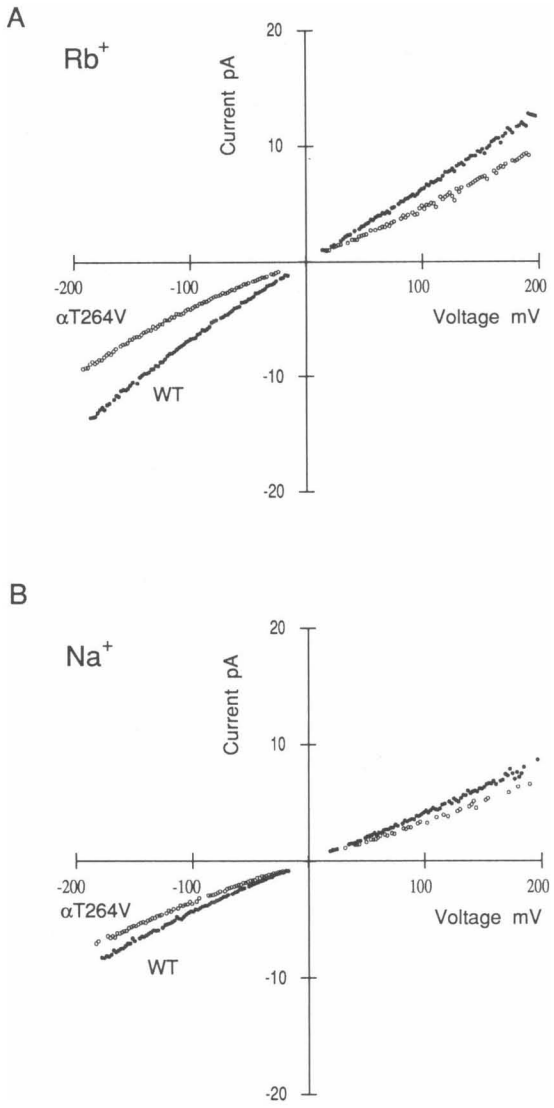

FIGURE 3 Comparison of current voltage curves of the wild type and αT264V mutant channel measured in 100 mM Rb^+ (*A*) and in 100 mM Na^+ (*B*). A large change in conductance and in the shape of the current voltage curve is found in Rb^+ (*A*) but only a small change in Na^+ (*B*). Channel current measured in either 100 mM Rb^+ (*A*) or 100 mM Na^+ (*B*), and 10 mM Hepes, and 10 mM EGTA, pH 7.2.

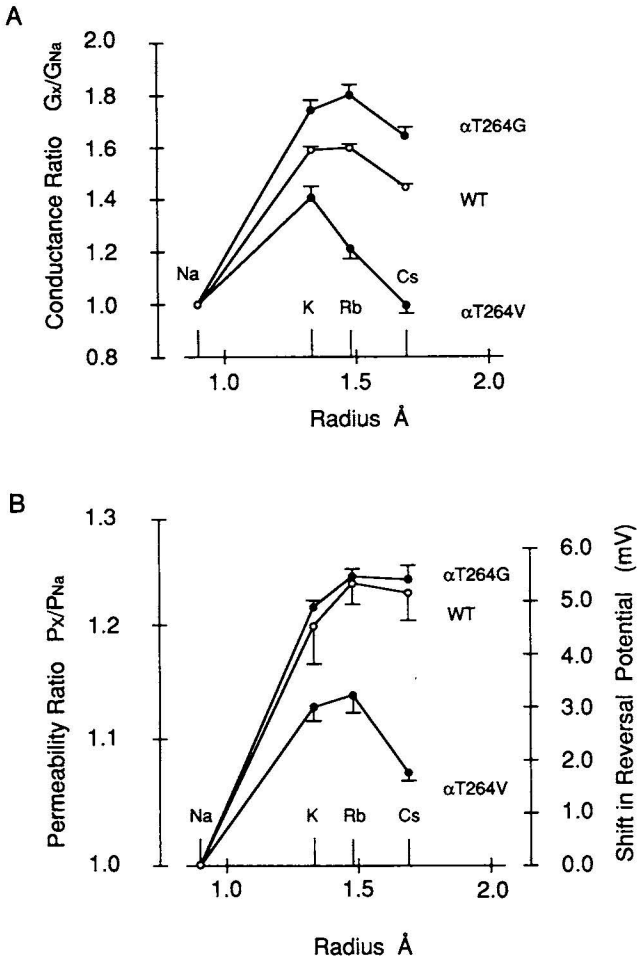

FIGURE 4 (*A*) conductance ratios for wild type (○), αT264V (●) and αT264G (●) mutant channel. Conductances normalized by Na^+ conductance of each channel are plotted against dehydrated ion radius. (*B*) permeability ratios measured by reversal potential in the wild type (○) and both αT264V (●) and αT264G (●) mutant channels plotted as a function of the dehydrated ion radius. The αT264V mutant channel has lower permeability for large ions in comparison to the wild type channel. The αT264G mutant channel has a higher permeability for large ions. Notice that the mutation αT264V also changes the selectivity sequence.

ments of selectivity lead to different selectivity sequences (Eisenman and Horn, 1983), we also examined the changes in selectivity defined by reversal potentials measured in biionic conditions. Reversal potentials and permeability ratios are presented in Table 2 and Fig. 4 *B*. The wild-type channel has the permeability sequence Rb > Cs > K > Na that corresponds to an Eisenman sequence II (Eisenman, 1962). The mutation αT264V decreases P_{Cs}/P_{Na} from 1.23 to 1.07 smaller changes are found for P_{Rb}/P_{Na} or P_K/P_{Na}. These differential changes turn the selectivity sequence of the αT264V mutant channel into an Eisenman sequence III. The αT264G mutant channel, on the other hand, shows small although noticeable increase in permeability ratios, not large enough to change the selectivity sequence of the channel.

Origin of conductance changes

To elucidate the mechanism that produces the changes in conductance we studied the conductance concentra-

TABLE 1 **Conductances of wild type, αT264V and αT264G mutant channels**

Channel	Ion	−100 mV conductance	100 mV conductance
		(pS)	(pS)
Wild type	Na^+	43.20 ± 0.26 (3)	40.74 ± 0.60 (3)
	K^+	68.78 ± 1.72 (10)	63.39 ± 2.37 (7)
	Rb^+	69.10 ± 1.58 (6)	63.37 ± 1.28 (6)
	Cs^+	62.52 ± 0.88 (4)	56.59 ± 0.90 (4)
	NH_4^+	85.91 ± 1.37 (3)	81.28 ± 1.52 (3)
αT264V	Na^+	35.46 (2)	34.01 (2)
	K^+	50.00 ± 0.90 (3)	46.70 ± 0.37 (3)
	Rb^+	43.04 ± 0.46 (5)	42.10 ± 1.67 (5)
	Cs^+	35.42 (2)	34.12 (2)
	NH_4^+	69.93 (2)	65.78 (2)
αT264G	Na^+	43.28 ± 0.67 (3)	40.52 ± 0.39 (3)
	K^+	75.61 ± 0.86 (6)	68.86 ± 1.98 (4)
	Rb^+	78.05 ± 1.00 (3)	72.08 ± 0.72 (3)
	Cs^+	71.17 ± 1.06 (3)	64.54 ± 0.73 (3)
	NH_4^+	91.43 ± 2.17 (3)	88.02 ± 1.47 (3)

Conductances measured in 100 mM permeant cation (NaCl, KCl, RbCl, CsCl, or NH_4Cl), 10 mM Hepes and 100 mM EGTA, pH 7.2, at 18 ± 1 °C. Data reported as mean ± SD (*n* is the number of observations).

tion relationship for Cs^+. This ion was chosen because the large differences in conductance found between wild type and mutant channels. The relation between conductance and Cs^+ activity for the wild-type, αT264V and αT264G mutant channels is shown in Fig. 5. The symbols represent conductance values measured at −100 mV. In comparison to the wild-type, the αT264V mutation produces a decrease in conductance in the whole

TABLE 2 **Reversal potentials and permeability ratios of wild type, αT264V and αT264G mutant channels**

Channel	Ion	V_{rev} ± SD	*n*	P_x/P_{Na}
		(mV)		
Wild type	K^+	4.54 ± 0.72	5	1.20
	Rb^+	5.35 ± 0.40	4	1.24
	Cs^+	5.16 ± 0.52	6	1.23
	NH_4^+	18.56 ± 0.53	4	2.10
αT264V	K^+	3.00 ± 0.26	5	1.13
	Rb^+	3.21 ± 0.33	3	1.14
	Cs^+	1.78 ± 0.15	4	1.07
	NH_4^+	22.98 ± 3.40	3	2.51
αT264G	K^+	4.89 ± 0.13	4	1.22
	Rb^+	5.47 ± 0.15	3	1.24
	Cs^+	5.42 ± 0.27	5	1.24

Reversal potentials measured in biionic conditions in 100 mM permeant cation (NaCl, KCl, RbCl, CsCl, or NH_4Cl), 10 mM Hepes and 100 mM EGTA, pH 7.2, at 18 ± 1 °C. The Na^+ solution was used as a reference in the intracellular side.

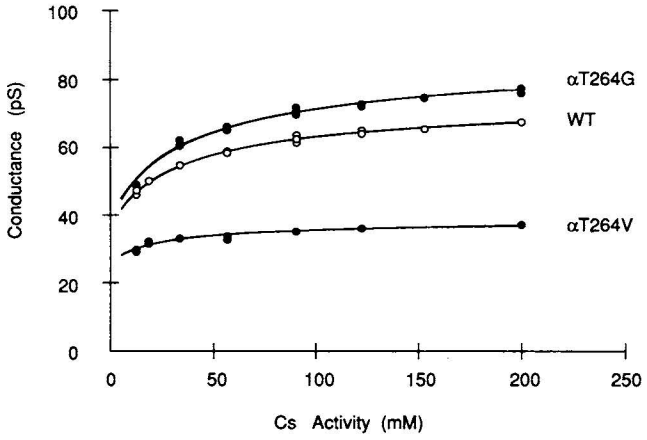

FIGURE 5 Saturation curves for wild type (○) and both αT264V (●) and αT264G (●) mutant channels. Smooth curves were calculated from the Eqs. 1 and 2 with the parameters shown in Table 3.

range of concentrations and it decreases the maximum conductance by about one half. In addition this mutant channel saturates at rather lower concentrations. The αT264G mutant channel on the other hand has a larger conductance, also in the whole range of concentrations. The steeper decrease of conductance at low ion concentrations suggest that saturation occurs at higher concentrations. The smooth lines in Fig. 5 are the result of the best fit to Eqs. 1 and 2. The curve for the wild-type channel was fitted using three free parameters, the maximum conductance, G_s, the apparent dissociation constant, K_s, and the surface charge density, expressed as R ($\sigma = e/\pi R^2$). The curves for the mutant channels were fitted using the surface charge density of 0.0623 charge per 100 Å^2 (R = 22.60 ± 0.98 Å), the value found for the fit of the wild-type channel curve. The parameters that give the best fit are presented in Table 3. Within the frame work of the simple model outlined above the mutation αT264V decreases G_s and K_s, whereas in the αT264G mutant channel both values are increased.

TABLE 3 **Estimations of K_s and G_s from saturation curves**

Channel	K_s	G_s	G_s/K_s	P_{Cs}/P_{Na}
	(mM)	(pS)		
Wild-type	28.3 ± 3.7	73.7 ± 0.9	2.60 ± 0.34	1.23
αT264V	14.4 ± 0.9	38.7 ± 0.3	2.69 ± 0.17	1.07
αT264G	34.1 ± 1.2	86.2 ± 0.6	2.53 ± 0.09	1.24

Parameters that produce the best fit of the saturation curves from Fig. 5 to Eqs. 1 and 2. The error is the standard deviation given by the program AJUSTE.

Absolute permeability and permeability ratios

Table 3 lists the ratio G_s/K_s, which is an estimate of the absolute permeability at zero ion concentration (Eisenman and Horn, 1983; Hille, 1984), for wild-type and the two mutant channels. No significant difference in the absolute permeability, estimated by this ratio, is seen. Probably this is due to the fact that K_s values are not accurately estimated as is G_s in the saturation curve which includes surface charge effects (Eqs. 1 and 2). However, possible differences in absolute Cs^+ permeability between the three channels can be derived in a model independent way considering the K_s values and conductance at low ion concentration. The estimates of the K_s values are > 10 mM bulk concentration, the lowest value of concentration for which conductance could be measured. In these low range of bulk ion concentration the conductances are likely to be proportional to the local ion concentration at the channel mouth, which is assumed to be the same for the different channels due to the fact that charged groups are the same in all three channels. The saturation curves (Fig. 5) show that at 10 mM Cs^+ bulk concentration, the conductance of the αT264G mutant channel is larger and the conductance of the αT274V mutant channel is smaller than that of the wild-type channel. This indicates an increase (αT264G) and decrease (αT264V) in absolute Cs^+ permeability, respectively, in the two mutant channels as compared to the wild-type channel. Thus, changes in Cs^+ current at low concentrations follow the changes in permeability ratio P_{Cs}/P_{Na} (Table 3).

Differences in Mg^{2+} sensitivity in mutant channels

One result obtained by the fit of the conductance saturation curves to the Michaelis-Menten model of ion transport in combination with surface charge is that in the αT264V mutant channel the amplitude of the elementary current saturates at lower concentrations than in the wild type channel. The opposite would be true for the αT264G mutant channel. Divalent ions like Mg^{2+} screen the effect of point charges, decreasing the local ion concentration and produce a reduction in channel conductance. Because the αT264V mutant channel saturates at rather lower bulk Cs^+ concentrations than the wild type channel the addition of Mg^{2+}, that reduces the local concentration at the channels' entry, should produce a smaller decrease in channel conductance. The αT264G mutant channel on the other hand saturates at higher Cs^+ concentrations. The addition of the same amount of Mg^{2+} should result in a larger decrease in channel conductance than in the wild-type channel. Conductances measured at −100 mV, in presence of 1 mM external Mg^{2+} for wild type and both mutant channels are presented in Fig. 6. In comparison to the wild-type channel the addition of 1 mM Mg^{2+} to the external face of the channel produces a larger decrease in the inward current through the αT264G mutant channel (28.2% compared to 21.4%). The αT264V mutant channel on the other hand is almost insensitive to Mg^{2+}, as only a 10% decrease in conductance was found. Thus, the different Mg^{2+} sensitivity of the conductance of the three channels is in agreement with the assumed changes in apparent dissociation constants for the mutant channel obtained from the fit of the saturation curves to the model.

DISCUSSION

The main goal of the work presented is to relate changes in primary structure, obtained by point mutations in the α-subunit, with changes in conduction properties of the AChR channel. We assume that the point mutations performed do not produce a global structural change of the channel protein. Several lines of evidence support this view. The replacement of αT264 by either valine or glycine produces a decrease and increase of channel current respectively and these conductance differences depend on the nature of the permeant ion being pronounced for Cs^+ and only slightly noticeable for Na^+. This is difficult to explain based on a nonspecific change in protein structure. Furthermore, the change in chan-

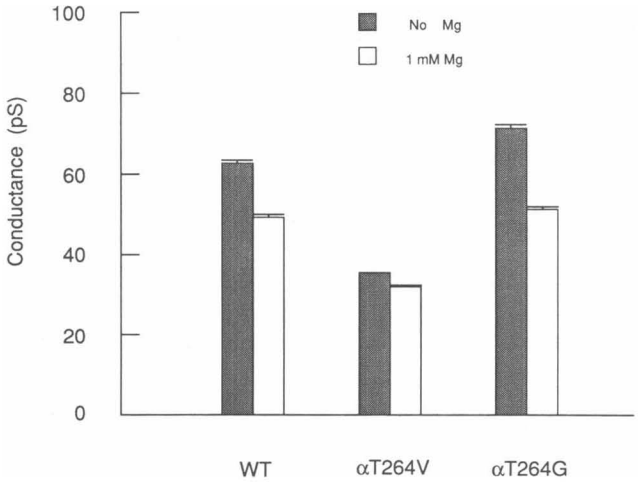

FIGURE 6 Differential sensitivity to Mg^{2+} of wild-type, αT264V and αT264G mutant channels. Conductance measured in 100 mM Cs^+, and 100 mM Hepes, pH 7.2 in absence of Mg^{2+} (*shaded bars*) and in presence of 1 mM external Mg^{2+}, (*white bars*). The applied potential was −100 mV.

nel current is inversely related to the volume of the amino acid residue that replaces αT264 (Villarroel et al., 1991). Other evidence that suggests that changes in the global structure of the channel are unlikely, comes from a comparison of structures observed in a protein family in which natural mutations do occur. Glycine and threonine are two amino acids residues located in the same topology of equivalent substitutions (Bordo and Argos, 1991). This suggests that the αT264G mutation is not likely to perturb the global structure of the protein. As for the αT264V mutation, valine is located in a close, but not equal topology of equivalent substitutions, and thus, is the best choice for a replacement of a threonine by an hydrophobic residue.

The selectivity of the AChR channel for cations has been examined by two criteria, conductance sequences and permeability ratios. Because the permeation process is rather more complicated than simple binding of ions to a single site, these two measurements often produce different answers (Eisenman and Horn, 1983) and is important to examine both.

In the AChR channel studied here, the best conducted ion is Rb^+ and not K^+ as in *Torpedo* AChR. The conductance sequence Rb > K > Cs > Na differs slightly from that of other AChR channels previously studied in frog muscle (Quartararo et al., 1987) or in Torpedo electric organ (Konno et al., 1991). The αT264V mutation produces a decrease in the conductance for large cations changing the conductance sequence to K > Rb > Cs > Na. The conductance for Na^+ is decreased by less than 20% in comparison to 60% decrease for Cs^+ ions. The αT264G mutation increases the conductance for large ions but not for Na^+ (Table 1) and does not change the conductance sequence. In summary, mutations in the position αT264 produce either a decrease or increase in conductance that is more pronounced for large ions, as if the size of the cavity left for ion movement were the determinant of current (Fig. 7).

The selectivity sequence from biionic reversal potentials was found to be Rb > Cs > K > Na for the wild-type channel. This sequence differs from both the conductance sequence and the aqueous mobility sequence thought to determine the permeation process. Because this sequence corresponds to sequence II of low field strength in the Eisenman series (Eisenman, 1962), one may conclude that the low field strength at the site of ion-channel interaction is not due to the ring of charged residues at the channels' entries but rather to the hydroxyl groups of the threonines in the α-subunits and presumably the hydroxyl groups of the homologous amino acid side chains from the other subunits (Fig. 1 *A*). The αT264V mutation, in addition to decreasing the channel conductance, also alters the permeability of the

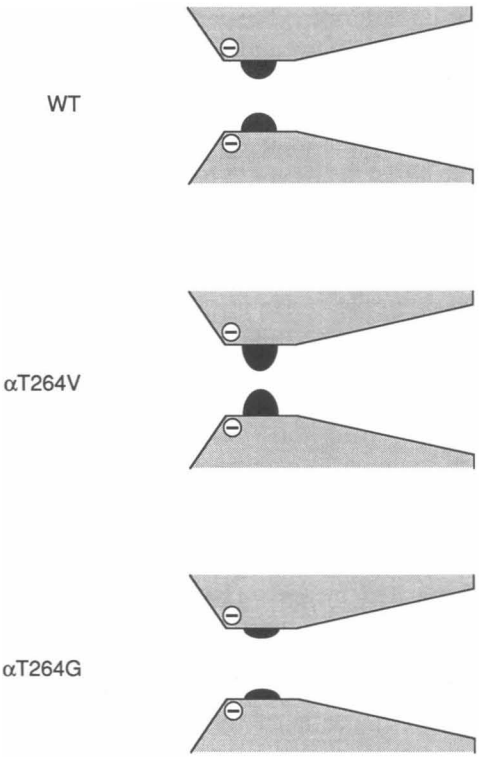

FIGURE 7 Relation between permeability and the nature of the amino acid residue located at position 264 in the α-subunit. The threonine residues of the two α-subunits contribute to the selectivity filter of the AChR channel. The ions are selected according to their sizes.

channel. In this mutant channel, the permeability ratio P_x/P_{Na} is decreased for large ions. This produces a change in the permeability sequence to Rb > K > Cs > Na, similar to the conductance sequence. The mutation αT264G also produces a small increase in P_x/P_{Na} for all ions, that is not large enough to invert the selectivity to sequence I in the Eisenman series, the aqueous mobility sequence. The effect of the αT264G mutation which decreases the size of the amino acid residue controlling the ion flow, is therefore to produce a less selective channel. This does not mean that the channel does not distinguish between ions but the selectivity resembles the aqueous mobility sequence. The αT264V mutation, which decreases the polarity of the amino acid controlling the ion flow, on the other hand makes the channel more selective.

A consequence of our interpretation of the conductance Cs-concentration curves is that the apparent dissociation constant of Cs^+ to the channel is decreased in the αT264V and increased in the αT264G mutant channel. It is important to test this hypothesis independently because the half saturating Cs-concentration

could not be determined experimentally. The different sensitivity of wild-type and mutant channels to Mg^{2+}, which neutralizes the charge effect of the anionic rings (Imoto et al., 1988) is not expected a priori because the present point mutations do not involve changes in charged residues. They could be interpreted as a consequence of the increase and decrease in the dissociation constant of the αT264G and αT264V mutant channels, respectively. Another indication of changes in ion binding comes from electrostatic calculations in a model of the M2 segment δ-subunit. A replacement of a ring of threonine by glycine residues results in the removal of an energy minimum from the energy profile (Eisenman et al., 1990). A feature worth being tested in such calculations is the role of an hydrophobic amino acid residue, placed in the narrow region of the pore, in the electrostatic energy profile.

In summary then, the fetal form of the AChR channel ions are selected by size. The role of the amino acid residues in the narrow part of the channel is to determine the size of the cavity left for ion movement. A mutation that decreases the size of that amino acid residue creates more space for ion flow and the conductance increases (Fig. 7). The release of the constraint for large ions shifts the selectivity towards the water mobility sequence. In the αT264V mutant channel, on the other hand, the ion flow and permeability for large ions decrease and the selectivity then moves away from the water mobility sequence in which Cs^+ is the most permeant ion. In this interpretation, a polar wide pore (water filled or containing hydroxyl side chain in its wall) will be Cs^+ selective whereas a polar marrow pore will be Na^+ selective. In addition to the size, the polarity of the amino acid residue in the selectivity filter may also be important because it influences ion binding. The role of polarity has not yet been studied systematically, as was done with the role of the size of the amino acid residues in the narrow region (Villarroel et al., 1991).

CONCLUSIONS

We have examined the conduction properties of the two αT264V and αT264G mutant channels. The former produces a decrease in both conductance and selectivity for large cations whereas the latter produces an increase in conductance and selectivity. Because the structure of the amino acid residues is known, the observation of an increase in conductance can be interpreted as the decrease in the apparent ion binding together with an increase in the free space for ion movement. As for the decrease in current found in the αT264V mutation result from the friction of large ions with the hydrophobic wall made by valine.

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DISCUSSION

Session Chairman: Adrian Parsegian *Scribes:* Marriella DeBiasi and Farzaneh Sorond

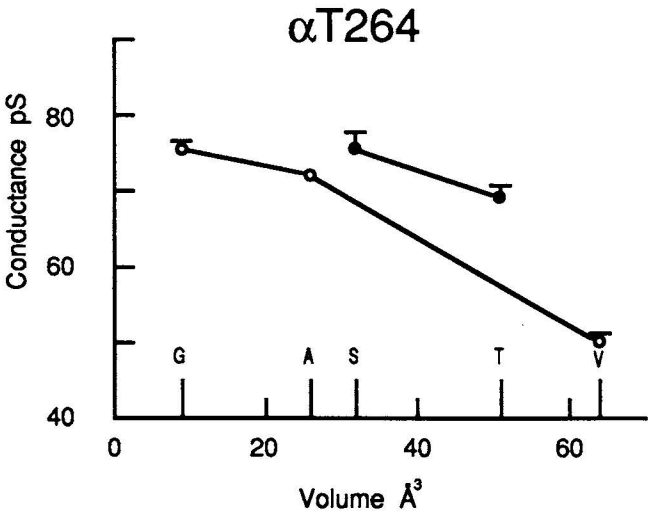
BURKHARD BECHINGER: Did you observe correlations between the volume of amino acid side chains and channel conductivity for the β, γ, or δ subunits?

ALFREDO VILLARROEL: I have measured the single-channel conductance in a variety of channels mutated at the αT264 residue. These conductances, when plotted against the volume of the mutated residue side chains, show an inverse relation in which large volume residues lead to lower conductances. However, I believe that there may be other important factors in addition to side-chain volume. The conductances of mutants with serine or threonine in αT264 residues are higher than one may expect from their side-chain volume.
I did replace "equivalent" positions in the M2 segment of other subunits, which include αT264, γT275, βG278, and δS279. Consistent with the αT264 mutations, I observed lower conductances when I replaced these residues with larger side-chain residues and higher conductances when I replaced them with smaller side chains. I'd therefore be inclined to suggest that this effect is not exclusively at the α subunit.

BECHINGER: Is there any evidence for specific interactions that you can identify (e.g., hydrogen-bonds, hydrophobic interactions) with the threonine residue?

VILLARROEL: Of course one cannot be sure, but I do think there is contact between the permeant ions and these groups in the polypeptide. As the plots of conductance versus side-chain volume illustrates, there are other factors, such as the presence of an −OH group in the side chain that we must consider.

ARTHUR KARLIN: Why look at the M2 helix as lining the channel pore? What should we think the function of Thr and Ser might be in the conduction of cations? According to Numa, there are four membrane spanning helixes with threonines and serines in M1 and M2. We know also that for cations to pass the channel they have to have oxygen to solvate them and some polar groups to substitute for water. Since chimeras made by Numa and Sackmann showed slight changes in conductance, and only when no calcium was present, I wonder which is the role of Thr and Ser under physiological conditions. Experiments from Changeaux's lab using the alleged open channel blocker chlorpromazine suggested that Ser and Thr are in the conduction pathway of this channel. But now we find that if you change conserved residues in a negatively charged region, e.g., if you mutate the Ser and Thr into Ala and Gly, the channel works better. Thus, why have these residues been conserved? Are we looking at the real function of these polar residues or is this an epiphenomenon?



VILLARROEL: I am not going to use blockers or labeling compounds. I think that because we are looking at the permeation phenomenon, we need to use permeant ions. The negative residues are also very important, especially if we consider their role in conduction and selectivity. The only thing that has not been shown is that you can increase the conductance through mutations on such residues. We should, however, be able to do this controlling some parameters such as, for example, the size or the polarity.

MARCO COLOMBINI: The alternating sequence present in the M2 region reminds me of a sided β-strand around Thr 264. There is a good alternating polar/nonpolar pattern, especially in the α helix. Have you thought about a β barrel model for the region forming the pore? Five β strands would form a pore that would be about the right size.

VILLARROEL: Yes, but here we are not proving the presence of α helices but just using this idea for our mutations. According to an α helix hypothesis the serine α S266 would be in the back part of the channel, and this is consistent with our results of mutations. Maybe in the future other experiments will invalidate this model.

COLOMBINI: If an α helix forms part of the pore, then its top part has to be totally nonpolar. Doesn't this bother you?

VILLARROEL: We know that hydrophobic blockers, such as QX222, can still bind to the channel. But if you look at the model of the channel, five Phe residues are facing the channel lumen and it is difficult for me to understand how this could be possible.

RONALD KABACK: I have a question about volume changes. Is it just the volume of the residue in 264 that is important? What happens with nonelectrolyte permeability?

VILLARROEL: It is not just the volume as you have seen before from the plots comparing hydrophobic residues with nonpolar ones in which changes in conductance are found. With respect to the nonelectrolytes, I have not looked at them so far.

RAJINI RAO: For a "systematic study" of the role of the size of the residues in the pore region, I would make more than two substitutions. Have you considered replacing Thr with other amino acids such as Ala and Ser or much larger residues such as tyrosine?

VILLARROEL: Yes, I have looked also at Ala and Ser. Not yet at Tyrosine.

RICHARD HORN: The I-V curve for the wild-type channel is linear, suggesting there is not a predominant rate limiting barrier for an ion to flow. It seems you have a series of small barriers through the membrane field. Since the selectivity is about the same at +100 or −100 mV, this suggests that either there is one rate limiting barrier that controls selectivity or all the barriers have the same selectivity. Do you think there is one rate limiting barrier and if there is, why are you getting a linear I-V?

VILLARROEL: I think there is one limiting barrier. Why I get a linear I-V, I don't know.

HORN: For αT264V, especially with large ions, you see a reduction in conduction; the I-V becomes superlinear. Do you think that the mutation is producing a large barrier in the middle of the electric field? Does the opposite happen for T264G? Another question is also related to the energy profile. You showed some kind of saturation. Where is the binding site? Is it different from the barrier?

VILLARROEL: The I-V curve for Gly is linear but I did not study this systematically. I think the binding site is near the cytoplasmic side.

HORN: Why?

VILLARROEL: A cluster of charged residues is near the cytoplasmic side causing me to think that this could be the binding site. In a barrier model you can locate this site near the cytoplasmic end of the channel too. If you then calculate the energy for an ion to pass through the channel you'll find an energy minimum right near the negatively charged residues.

HORN: Does this model produce a linear I-V relation?

VILLARROEL: The barrier model, no. The electrostatic model, I don't know.

KARLIN: If the negative charges are functioning as a binding site, then why under physiological conditions, that is, in the presence of Ca, do mutations have no effect on the permeability of ions? It is hard to see what would be the appropriate function of these residues under physiological conditions.

Furthermore, the intermediate ring was the narrowest part of the channel, equally accessible from both sides of the membrane because Mg could affect what happened in that site. But now the barrier has been moved around alpha T264. Would this change the interpretation of the previous results?

VILLARROEL: Yes, the rat barrier has been moved with respect to the *Torpedo* barrier. With respect to Mg, I wanted to test it under simple conditions, with one permeant ion, without Ca.

KARLIN: What bothers me is the question of what maintains the structure? Where is the evolutionary value of this conservation given your observation that mutations do not seem to have a physiological effect.

OLAF ANDERSEN: First, systematic studies on the effect of amino acids geometry and polarity on channel function have been done for many years in the gramicidin channel.

Second, I agree with Dick, but from a different point of view. The term "selectivity" filter implies that there is a spatially-limited region of the pore where the selection occurs. This is really hard to reconcile with the linear I-V curves you find. I really doubt you have a selectivity filter in the Hillean sense.

T264V alters the binding constant, suggesting that the Val is involved in the binding site. This suggests that you may be dealing with a well instead of a barrier and that you are not touching the selectivity filter (barrier).

Third, it would be nice if you would calculate the intrinsic binding constants relative to the hypothetical state where no surface potential is present. That would provide information about the intrinsic ion-channel interaction.

You speak of changes in volume, but there are not great changes in volume between Thr and Val. Furthermore, if you look at the geometry, you will have to consider the hydrogen bonding interactions. For Thr you will need a more sophisticated volume measurement (cf Hille, 1972). Furthermore, when you compare Val and Thr in the alpha subunit and in the delta subunit, you compare different situations because in one case you have one Val and in the other, two. In any case, if you are changing the selectivity filter, what happens to divalent cations? Do you alter their permeability? This seems to be the most sensitive test of whether the OH group are involved in the selection of ions or not.

VILLARROEL: We did not look at permeability of divalent cations.

ADRIAN PARSEGIAN: Size, charge, polarity, and hydrophobicity are all elements that people are thinking about as determining selectivity, but we are not able to distinguish yet. Is the thought that putting in a divalent cation will allow you to distinguish between size and charge?

VILLARROEL: We always think about models, but what we find is a complicated protein. We find new phenomena that go beyond what we might predict using models.

MICHAEL GREEN: Have you looked at the pH effect?

VILLARROEL: No, we did not want to change the charge. We studied only neutral residues.

ROBERT GUY: You mentioned that the Phe were a problem. Aromatics are hydrophobic but this does not mean they are apolar. The role of aromatics and π-electrons may not be completely appreciated.

With respect to your idea of a narrowest part of the channel, do you have experimental evidence that the residues further up on the M2 region are not equally controlling ion selectivity and permeability properties of the channel.

VILLARROEL: I have tested αS266 and αS268 further up in the α subunit. I have not seen changes in conductance when I substitute these residues by Ala.

GUY: Others have postulated that the pore has a cone shape which is consistent with your interpretation.

VILLARROEL: I did not say I believe that, only that I use that concept.

GUY: If it does, M1 may constitute part of the wall pore. Are there any studies to test this hypothesis?

VILLARROEL: Yes. Experiments in which the M1 segment between gamma and epsilon subunits were changed showed no change in channel conductance.

GORDON RULE: The types of mutations that you're making change the H-Bonding potential and volume of side chains. These can affect helix packing. Can spectroscopic measurements detect changes in helix packing?

VILLARROEL: We think the packing hasn't changed.

RULE: Is there any evidence that you can change the orientation of the helix.

VILLARROEL: No.

DAVID BUSATH: Olaf Andersen makes a good point that the reduction of Cs conductance is due to a barrier. A barrier increase would make more sense than a change in affinity to explain the change in selectivity. On the other hand, you pointed out that Tris can get through. Then there is clearly a large volume of H_2O between one turn of the α helix and the next. I calculated between 6–8 H_2O molecules. It's hard to imagine how such a large change in volume can produce a narrow constriction. We have the same dilemma with the K channel of Arthur Brown's group. Changing Val to Leu changes the selectivity. Any thoughts how your results relate to theirs, and to how such a big volume could provide a barrier change?

VILLARROEL: This also bothered me. How can an ion the size of Tris be permeant? Is it that the channel is soft and can expand and pass Tris? Perhaps the channel is not such a rigid structure. However, it is hard to reconcile how the channel could be flexible for large ions only.

BUSATH: From my molecular modeling studies, if you take five α helices and pack them, it is hard to get a constriction much narrower than $\sim 7 \times 9$ Å without having large side chains projecting into the pore. If you've just Thr and Ser, you'll have a fairly large hole. Based on models, if you have 10-stranded β barrel structures, you will have a big area, and it will be difficult to produce a narrow constriction without severe contortions of the backbone. Are we at the stage that we have to suppose that there must be a constriction and severe contortions in the backbone structure?

VILLARROEL: I think so.

BUSATH: I don't. There are other interpretations for the selectivity data.

PARSEGIAN: Do we know enough about what selectivity means? Based on gramicidin results, it is hard to understand how selectivity comes about.

ALAN FINKELSTEIN: With regard to selectivity, in this model, is it true that the part of the channel that crosses the membrane is not charged?

VILLARROEL: No. I said that the M2 segment is within the membrane and the charges on the intermediate ring are in the middle of the M2 segment. I don't know about the rest of the channel. Of course there can be changes inside the pore.

FINKELSTEIN: I'm talking about the lining of the pore. According to the model, there are no charges lining the pore. Is that a fair statement?

VILLARROEL: Yes.

FINKELSTEIN: Are people happy with small divalent cations (e.g., Mg and Ca). Is it energetically favorable to cross the pore without any charges lining it? The examples that I know are the gramicidin channel and the nystatin channel which are impermeant to divalent cations.

VILLARROEL: Are you comparing AChR with gramicidin channels?

FRANCISCO BEZANILLA: The AChR channel is a BIG hole.

VILLARROEL: Yes. It can pass Tris.

FINKELSTEIN: Let's assume an 8-Å diameter. Don't you think that there is an energetic problem?

PARSEGIAN: Not prohibitive. The pore is wide enough and the "filter" short enough to allow charges to pass.

RAO: If there were negative charges inside the pore, the cation would bind tightly. Therefore, the charges lining the pore might act like a barrier to ion flux.

PARSEGIAN: Yes. There is always the question why a selective binding site might not actually inhibit the flux.

GREEN: If there are any charges at the end of the channel that allow Mg to get through, once it gets to that end of it, there will be an entropy effect that will help it to leave; there are many more configurations for the ion in the solution outside.

KASIANOWICZ: You presented mutations that result in sensible changes to the conductance. Did any mutation give surprising or paradoxical results? That is, do any point mutations work the "wrong way" within the framework of your working hypothesis (that α T264 residue side-chain volume sterically regulates conductance)?

VILLARROEL: I have some mutations that show no effect on channel conductance, but so far I have not found paradoxical results.

B. VEERAPANDIAN: I'd like to share some ideas on Phe mutations and the shape and function of the side chain. We have a β barrel structure with three Phe inside the barrel and one at the open face of it. If you mutate Phe within the barrel to other residues with large hydrocarbon side-chains, the activity does not change very much, but if you mutate the Phe at the tip of the protein, then the IL-1 activity is very much altered. The role of the π-electron may be critical for binding activity. About the shape of the side-chain, if you mutate the arginine on the tip of the barrel to Gly or Ala the protein is not active. Above Ala, whatever you mutate, all are active. The location of the residue and the size of the side-chain seem to play a critical role in the activity of the protein.

ANDERSEN: If you look upon the selectivity sequence of the wild-type, it looks as if it is the hydrated ion that diffuses. Now when you mutate the Thr, and change the side-chain bulk, you come to a point where you do not change the van der Waals volume very much, but you see a dramatic decrease in the sequence of the permeabilities (Val versus Thr). This suggests that instead of focusing on the volume you should look at the hydroxyl groups.

VILLARROEL: I think that we have to say that there is something more in addition to the volume, because among Val, Gly, and Ala the only difference is the size of the side-chain and not the presence of a hydroxyl group. So volume is important but it is not the only factor.